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PRINCIPAL INVESTIGATOR: Linda deGraffenried, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Health
Science Center at San Antonio
San Antonio, Texas 78284-7828

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FOREWORD

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MAIN REPORT:

ABSTRACT:

Studies have shown that elevated expression levels of estrogen receptor α (ER α) in benign breast epithelium is a risk factor for progression to invasive breast cancer, and that breast tumor tissue expresses elevated levels of ER α as compared to adjacent normal tissue. Previous studies suggest that ER expression is partly regulated at the transcriptional level. The research funded by this grant has focused on investigating the factors that regulate the expression of ER α in breast cancer cells as potential targets for clinical therapy.

The ER α minimal promoter is contained within the -245 bp to +212 bp region of the gene, and contains an E box, a GC box, and a CA rich region that are critical for ER α promoter activity. A multi-protein complex containing Sp1/Sp3, ER α and USF-1 interacts with the ER α minimal promoter. Independently, Sp1 and USF-1 are able to each transactivate the ER α minimal promoter, but together have an additive, if not synergistic, effect on transactivation. Additionally, a short sequence (AACT) located at -203 bp to -200 bp appears to be essential for functional activity since mutation of these nucleotides results in loss of all transcriptional activity of the promoter. The results of these experiments could prove clinically important in identifying new treatment targets.

INTRODUCTION:

Understanding the molecular changes that occur during the progression of breast disease will provide clinicians with both earlier markers for detection of the disease, as well as potential targets for early intervention. One well-recognized target is the estrogen receptor α (ER α). We know that ER α is expressed at elevated levels in many tumors as compared to the adjacent normal tissue (1, 2, 3). In fact, studies have shown that elevated levels of ER α in benign breast epithelium is itself a risk factor for progression to invasive breast cancer (4, 5). The ER is a nuclear transcription factor and a well known mitogen for breast cancer cells (6), thus its overexpression may play a permissive role in the progression of breast cancer (7). We hypothesize that the delineation of the mechanisms regulating ER α expression is clinically important and may identify new treatment targets. We plan to address the identification of these potential new targets with the following **Specific Aims**:

1. To identify regions within the ER α promoter regulating ER α transcription in breast cancer cells (months 1-12).
2. To identify factors binding to transcriptional regulatory regions of the ER α promoter (months 12-30).
3. To determine the relevance of candidate proteins in the regulation of ER α transcription in breast cancer cells (months 18-36).

BODY:

I have made excellent progress in this final year of my investigation, focusing predominantly upon Specific Aims 2 and 3 and am on schedule in the grant. The work done in the first year of this study led to the identification of three transcriptional regulatory regions within the ER α minimal promoter, including a GC box, a flanking imperfect E box and a CA rich region, as well as the finding of Sp1/Sp3 as critical regulatory factors for the expression of ER α in breast cancer cells (Appendix A). The work done in the proceeding two years has focused on identifying other transcription factors that regulate ER α expression in breast cancer cells.

To identify other transcription factors that interact with the ER α minimal promoter (**Specific Aim 2**), we used a combined EMSA, Western blot analysis technique. Briefly, we probed whole cell extracts from MCF-7 breast cancer cells with a 32 P-labeled oligonucleotide that spanned the ER α minimal promoter from nucleotides -245 to -182 (8). After separation through a 5% acrylamide gel and exposure to x-ray film, the protein/DNA complex (box, Fig. 1A) was excised from the gel and resolved (Fig 1B, lane A), along with whole cell extract from MCF-7 cells as a positive control (Fig 1B, lane B), through a 10% SDS-polyacrylamide gel. A standard Western blot protocol was followed as described previously (9). We probed the Western blot containing the minimal promoter binding proteins with antibodies to Sp1 (Fig 1B, Sp1) and Sp3 (Fig 1B, Sp3), which we had previously identified as critical for ER α transcription. A non-consensus E-box at -231 to -226 bp, which is a potential binding site for the basic helix-loop-helix transcription factor USF-1 (10), had been identified in the first year of the study as critical for full transcriptional activity of the ER α promoter, so we also probed the blot with an antibody to USF-1 (Santa Cruz Biotechnology, Santa Cruz, CA) (Fig 1B, USF-1). In addition, previous work done in the lab had identified the ER itself as able to autoregulate itself through the minimal promoter (11). We therefore also probed the blot with an antibody to ER (Fig 1B, ER). Antibodies for AP2 and GATA-3 were also used, but were negative in the EMSA lane, but positive in the MCF-7 lane (data not shown). This demonstrates that Sp1, Sp3, ER α and USF-1 all interact with the ER α minimal promoter.

In order to better understand the dynamics of the interaction of the multi-protein complex containing Sp1/Sp3, USF-1 and ER α and the ER α minimal promoter, we performed a series of EMSAs using the minimal promoter probe described earlier, and non-radioactive *in vitro* translated (IVT) proteins for Sp1, Sp3, USF-1 and ER α (Fig 2.). IVT products were prepared using the TNT[®] Coupled Reticulocyte Lysate System (Promega, Madison, Wi) as per manufacturer's instructions. The expression plasmid for the ER α has already been used in our lab for EMSA (11). We obtained a CMV-driven expression plasmid for Sp3 from

Invitrogen (San Diego, Ca). The expression plasmid for USF-1 (pCX-USF-1) was kindly provided by Robert G. Roeder (12), while an Sp1 mammalian expression plasmid was kindly provided by Paul D. Gardner. In Figure 2, lanes A and B are controls, containing only the ER α minimal promoter probe, and probe with rabbit reticulocyte, respectively. Lanes C-G contain the USF-1 IVT product. In lane C a specific DNA/protein complex is formed (star) which is disrupted by the addition of an antibody to USF-1 (Santa Cruz Biotechnology) (lane D), but not by the addition of rabbit pre-immune serum (lane E). The addition of 10-fold molar excess of a cold consensus USF-1 oligonucleotide (5' CACCCGGTCACGTGGCCTACACC 3') in lane F is able to compete with the complex, but the same molar excess of an Sp1 consensus sequence (5' AATTCGATCGGGGCGGGGCGAGC 3') in lane G is not. Lanes H-L contain the Sp1 IVT product. Again, a specific complex is formed in lane H (star), which can be supershifted (arrow) by the addition of an Sp1 antibody (lane I). The complex is not disrupted by the addition of the rabbit pre-immune serum (lane J) or the USF-1 consensus oligonucleotide (lane L), but is competed by a 10-fold molar excess of the cold Sp1-consensus oligonucleotide (lane K). The same is true for the Sp3 IVT product in lanes M-Q. A specific complex is formed (star, lane M) which can be supershifted with an antibody to Sp3 (lane N, arrow), or competed with by the Sp1 consensus oligonucleotide (lane P). The complex is unaffected by the addition of either rabbit pre-immune serum (lane O), or the USF-1 consensus oligonucleotide (lane Q). In contrast, the ER α IVT product is unable to bind to the ER α minimal promoter probe (lanes R-S), as we have seen previously in this lab (11). The ER α IVT product is able to bind to a consensus ER sequence (11) (free probe, lane T), since incubation of the ER α product with this oligonucleotide results in the formation of a specific complex (lane U, star), which can be supershifted by the addition of an ER antibody (lane V, arrow).

These sets of experiments demonstrate that Sp1, Sp3 and USF-1 are all able to bind to the ER α minimal promoter, yet the ER α itself is not. This suggests that ER α must be

part of the complex through protein:protein interactions. This has been demonstrated on other promoters, most notably through interaction with Sp1 at Sp1-binding sites (13) (14).

To evaluate the effect of USF-1 on ER α promoter transcriptional activity (**Specific Aim 3**), we performed co-transient transfections assays into MCF-7 breast cancer cells using the ER α minimal promoter luciferase reporter described in earlier work (see Appendix A, fragment A), the pCX-USF-1 expression plasmid, and an expression plasmid for a dominant-negative USF-1 which is void of the DNA-binding domain (Δ bTDU1), which was generously provided by Anne-Marie Le francois-Martinez (15). The plasmid pNull-Renilla (Promega) was co-transfected for transfection normalization. All transfections are reported as fold activity over control after normalization for Renilla expression; with the control being the ER α minimal promoter luciferase reporter co-transfected with the pCDNA3.1 vector (Invitrogen) (Fig 3, column A). As shown in Figure 3, increasing exogenous expression of wild-type USF-1 increased promoter activity two to five fold over control, (Fig 3, columns B-D, respectively). Co-transfection with the dominant-negative USF-1 reduced wild-type USF-1 transactivation of the minimal promoter in a dose-dependent manner, 30% (column E) to 41% (column F). This data demonstrates an important role for USF-1 in the regulation of ER α transcription.

To further understand the significance of Sp1, USF-1 and ER α on transcriptional activity of the ER α minimal promoter (**Specific Aim 3**), we again performed co-transient transfection assays into MCF-7 breast cancer cells. We transfected the ER α minimal promoter luciferase reporter along with either the expression plasmid for Sp1, the expression plasmid for USF-1, the expression plasmid for ER α , or a combination of the expression plasmids, along with the pNull-Renilla plasmid for transfection normalization. The results are reported as fold over control after normalizing for Renilla expression (Fig 4.). Expression of Sp1 alone (Fig 4, column B) had little effect on promoter activity, as is commonly seen with mammalian cell transfection of Sp1. Expression of USF-1 (column C) resulted in a six-fold increase of activity over control. ER α alone had little effect on activity

(column D). When USF-1 and Sp1 are combined, as in column E, a synergistic effect is observed, with a thirty-fold increase in activity. The combination of ER α with either Sp1 (column F) or USF-1 (column G) appears to augment the activity of these factors, with activity at three- and fourteen-fold levels over control, respectively. When all three are expressed together (column H), activity is at its greatest, with a forty-eight-fold level of activity over control. This would suggest that while important individually, it is in their combination that these factors are able to most affect transcriptional activity of the ER α minimal promoter.

The CA rich region of the ER α minimal promoter, located between nucleotides -203 to -192 was critical for ER α transcriptional activity in earlier studies (see Appendix A, Fig 2B, fragment E). EMSA using a probe specifically to this area (-212 bp to -182 bp (16)) failed to detect any binding, and mutation of this region did not interfere with binding by the Sp1 family members or USF-1 to their upstream binding sites (data not shown). Therefore, to better understand the functional significance of this region, we introduced sequential 4 bp mutations into the CA rich region of the promoter. These reporter constructs were then transfected into MCF-7 cells and the results were expressed as fold activity compared to control, which was the pGL3-Basic vector (Promega), after normalizing for Renilla expression (Fig 5). The wild-type ER α minimal promoter fragment showed about a six-fold level of activity over control (Fig 5, A). The EcoRI-mutated -203 to -192 fragment (Fig 5, E) demonstrated an 80% loss of activity compared to the wild-type fragment. Mutation of nucleotides -203 to -200 (AACT) also resulted in complete loss of promoter activity (Fig 5, E-1), while mutation of the flanking 3' sequences had little effect on activity (Fig 5, E-2 and E-3). Earlier work (Appendix A, Fig 2B, fragment D) demonstrated that mutation of the flanking 5' sequence also has little effect. Ongoing DNase I footprinting analyses of this region will hopefully demonstrate whether the functional importance of this element is due to binding by transcription factors that are not directly recruited to the promoter at this

specific sequence, but are recruited as part of a protein complex. Whatever the reason, the integrity of nucleotides -203 to -200 is essential for ER α promoter transcription.

Transcriptional regulation of any promoter is usually complex. This appears to also be the case with the regulation of the ER α promoter. The data contained within this study lay a strong foundation for further work in understanding the mechanisms regulating ER α expression in breast cancer and developing potential clinical targets focusing on the transcriptional events which are involved in the progression of the disease.

KEY RESEARCH ACCOMPLISHMENTS:

- Defining a critical regulatory region of the ER α promoter responsible for a majority of the transcriptional activity of the gene.
- Determining that Sp1 and/or Sp3 are critical for ER α transcription in breast cancer cells. These are the first transcription factors, other than the ER itself, which have proven to have any regulatory effect on ER α expression.
- Demonstrating that ER α transcription is regulated by a multi-protein complex which includes, Sp1/Sp3, USF-1 and ER α , and showing that when expressed together, have a synergistic effect on ER α promoter activity.
- Defining a critical function for the nucleotides located between -203 and -200 of the ER α gene in the regulation of ER α transcription.

REPORTABLE OUTCOMES:

Manuscripts:

SP1 IS ESSENTIAL FOR ESTROGEN RECEPTOR α GENE TRANSCRIPTION. Linda A. deGraffenried, Susan G. Hilsenbeck, and Suzanne A. W. Fuqua. Journal of Steroid Biochemistry and Molecular Biology (submitted)

Abstracts:

deGraffenried LA and Fuqua SAW. Identification of regions involved in transcriptional regulation of the estrogen receptor gene in human breast cancer cells.. 20th Annual San Antonio Breast Cancer Symposium. Breast Cancer Res Treat. Oct: 46(1):22-113, 1997.

deGraffenried LA and Fuqua SAW. Analysis of the role of the transcription factor Sp1 in transcriptional regulation of the estrogen receptor gene in human breast cancer. 8th Annual Symposium on Cancer Research in San Antonio. July 17, 1998.

deGraffenried LA and Fuqua SAW. The Sp1 transcription factor is involved in transcriptional regulation of the estrogen receptor gene in human breast cancer cells. 21st Annual San Antonio Breast Cancer Symposium. Breast Cancer Res Treat 50(3):333, 1998.

deGraffenried LA, Welshons WV, Curran EM, Fuqua SAW. Transcriptional regulation of the estrogen receptor gene minimal promoter in human cancer cells. 81st Annual Meeting of the Endocrine Society. P1-280(194), 1999.

deGraffenried LA, Hopp TA, Lee AV, and Fuqua SAW. A multi-protein complex including the estrogen receptor (ER) and USF-1 is involved in the regulation of the ER gene minimal promoter in human breast cancer cells. 22nd Annual San Antonio Breast Cancer Symposium. Breast Cancer Res Treat 57(1):96, 1999.

Degrees obtained:

Institution	Molecular Medicine program University of Texas Health Science Center, San Antonio
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CONCLUSION:

The work that has been accomplished by this study is significant. The ER α minimal promoter is located within the -245 bp to +212 bp region of the gene and contains a majority of the activity of the promoter in breast cancer cells. A multi-protein complex, containing at least Sp1/Sp3, USF-1 and ER α interacts with the minimal promoter and is important for the regulation of ER α transcription in breast cancer cells. Additionally, the integrity of a short 4-bp sequence within the minimal promoter is essential for transcription of the promoter. Together, these data present a strong foundation for the advancement of further studies that target specific transcriptional events that occur in the progression of breast disease.

REFERENCES:

1. J. C. Allegra, et al., *Cancer* **44**, 228-31 (1979).
2. G. J. Netto, et al., *Am J Clin Pathol* **94**, 14-7 (1990).
3. T. van Agthoven, M. Timmermans, J. A. Foekens, L. C. Dorssers, S. C. Henzen-Logmans, *Am J Pathol* **144**, 1238-46 (1994).
4. S. A. Khan, M. A. Rogers, J. A. Obando, A. Tamsen, *Cancer Res* **54**, 993-7 (1994).
5. S. A. Khan, M. A. Rogers, K. K. Khurana, M. M. Meguid, P. J. Numann, *J Natl Cancer Inst* **90**, 37-42 (1998).
6. M. A. Fanelli, et al., *Breast Cancer Res Treat* **37**, 217-28 (1996).
7. B. E. Henderson, L. Bernstein, in *Diseases of the Breast* J. R. Harris, M. E. Lippman, M. Morrow, S. Hellman, Eds. (Lippincott - Raven, Philadelphia - New York, 1996) pp. 185-200.
8. S. A. Fuqua, et al., *Breast Cancer Res Treat* **32**, 177-86 (1994).
9. A. K. Tandon, G. M. Clark, G. C. Chamness, A. Ullrich, W. L. McGuire, *J Clin Oncol* **7**, 1120-8 (1989).
10. R. A. Rippe, A. Umezawa, J. P. Kimball, M. Breindl, D. A. Brenner, *J Biol Chem* **272**, 1753-60 (1997).
11. C. G. Castles, S. Oesterreich, R. Hansen, S. A. Fuqua, *J Steroid Biochem Mol Biol* **62**, 155-63 (1997).
12. A. L. Roy, et al., *Embo J* **16**, 7091-104 (1997).
13. R. Duan, W. Porter, S. Safe, *Endocrinology* **139**, 1981-90 (1998).
14. C. Vyhlidal, I. Samudio, M. P. Kladde, S. Safe, *J Mol Endocrinol* **24**, 329-38 (2000).
15. A. M. Lefrancois-Martinez, A. Martinez, B. Antoine, M. Raymondjean, A. Kahn, *J Biol Chem* **270**, 2640-3 (1995).
16. S. Green, et al., *J Steroid Biochem* **24**, 77-83 (1986).

APPENDIX A

SP1 IS ESSENTIAL FOR ESTROGEN RECEPTOR α GENE TRANSCRIPTION

Linda A. deGraffenried¹, Susan G. Hilsenbeck², and Suzanne A. W. Fuqua^{2*}

¹ Division of Medical Oncology, The University of Texas Health Science Center, San Antonio Texas 78229

² Baylor College of Medicine, Breast Center, One Baylor Plaza, Houston Texas 77030

* To whom all correspondence should be addressed

Baylor Breast Center

Baylor College of Medicine

One Baylor Plaza

Alkek 1220, MS: 600

Tel.: 713-7981600;

Fax: 7130798-1673

E-mail: sfuqua@bcm.tmc.edu

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Influence of Sp1 on ER α Gene Transcription

SUMMARY

The exact molecular mechanisms regulating estrogen receptor (ER) α expression in breast tumors are unclear, but studies suggest that the regulation is at least partly transcriptional. We therefore undertook a detailed analysis of ER α promoter activity in a number of breast cancer cell lines. We find that the majority of ER α promoter activity lies within the first 245 bp of the 5'-flanking region of the gene. Three elements essential for full ER α promoter transcriptional activity were identified within the -245 bp to -192 bp region in transient transactivation assays using linker-scanner mutation analysis. These three elements include two binding sites for the Sp1 family of transcription factors as well as a non-consensus E box. We show that both Sp1 and Sp3 bind to this region using electrophoretic mobility shift assays. Exogenous expression of Sp1 or Sp3 in Sp1/3-negative *Drosophila* Schneider SL2 cells results in transactivation of the -245 bp to +212 bp fragment of the ER α promoter. These data demonstrate that transcription of ER α is dependent upon the expression of members of the Sp1 family.

Influence of Sp1 on ER α Gene Transcription

1. Introduction

Understanding the molecular changes that occur during the progression of breast disease could provide clinicians with earlier markers for detection of the disease, as well as potential targets for early intervention. One well-recognized target is ER α . We know that ER α is expressed at elevated levels in many tumors as compared to the adjacent normal tissue [1-3]. In fact, studies have shown that elevated ER α in benign breast epithelium is itself a risk factor for progression to invasive breast cancer [4,5]. The ER is a nuclear transcription factor and a well-known mitogen for breast cancer cells [6], so that its overexpression may play a permissive role in the progression of breast cancer [7].

ER α expression is regulated, in part, at the mRNA level both in human breast cancer cell lines and in tumors [8-12]. The mechanisms regulating this expression so far remain unclear. There are at least two major transcription start sites residing within the upstream promoter region that have been identified in human breast cancer cells [13,14]. One of these, the P1 start site, is located at the +1 nucleotide relative to the cap site, and a less utilized P0 site is located at -3090 bp relative to the major transcription start site. Recently, a number of other potential start sites have been reported [15,16]. However, the relevance of these multiple start sites to the elevated levels of ER α expression seen in breast tumors is not understood at present.

Other investigators have identified potential regulatory regions in the ER α promoter [17,18]. One enhancer region, termed ER-EH0, contains an AP-1 binding site as described by Tang *et al* [17]. Although it has not yet been shown whether AP-1, or any other factor, binding to this enhancer region can directly transactivate the ER α promoter, it has been demonstrated that this region contains stronger promoter activity in ER-positive than in ER-negative breast cancer cells. Another candidate regulatory element has been identified within the 5' untranslated region [18], but mutation of this proximal region suggests that it has a minimal effect on the ER-EH0 enhancer activity [17]. It has also been shown that this proximal region binds the factor ERF-1, now known to be AP2 γ [19]. AP2 γ appears to be more highly expressed in ER-positive cell lines [18], but studies investigating how expression of AP2 γ affects ER α promoter activity have not been detailed [20]. Recent studies in tumors would suggest that while there is a positive correlation between AP2 γ expression and ER α expression, ER α expression is not solely dependent upon AP2 γ expression, as evidenced by the fact that some ER-positive tumors fail to express AP2 γ [21]. Obviously, further work is required to fully determine AP2 γ 's role in the regulation of ER α transcription.

In the current study, we have identified key regulatory elements essential for ER α transcription by examining the activity of ER α promoter fragments from -4100 bp to +212 bp in a panel of breast cancer cell lines.

Influence of Sp1 on ER α Gene Transcription

We show that the majority of ER α promoter activity lies within the -245 bp to +212 bp region. We also demonstrate important roles for three distinct elements within this region of the promoter and the 5' untranslated region. Two of these are potential sites for binding of Sp1 transcription factors, and we show that exogenous expression of either Sp1 or Sp3 increases ER α promoter activity. Furthermore, inhibition of DNA binding of these two transcription factors results in a decrease of both ER α promoter activity and ER α expression in breast cancer cells. We conclude that the Sp1 family of transcription factors play an essential role in ER α gene transcription.

Influence of Sp1 on ER α Gene Transcription

2. Materials and Methods

2.1. Plasmid Construction

To isolate a genomic clone containing the 5' flanking region and first exon of ER α from a bacterial artificial chromosome library of human male fibroblasts CCD-978SK (ATCC No. CRL 1905) [22], Touch polymerase chain reaction (PCR) amplification [23] using primers ⁻²¹⁴⁶TGAGCAGACA GCAAGTCTCC⁻²¹²⁶ and ⁻¹⁹⁴¹TGGCTCAGTG TGTGAACTAG G⁻¹⁹⁶² to the ER α gene [14] was used. For Touch PCR, the genomic DNA was amplified in a 25- μ l reaction volume containing 100 pmoles of each primer as well as 5 units of Amplitaq polymerase (Perkin-Elmer, Foster City, Ca), 320 μ M dNTPs, and 3 mM MgCl₂, using the following parameters: 15 sec at 96⁰ C, 30 sec at 55⁰ C and 3 min at 72⁰ C for 35 cycles followed by an extra cycle with a 10-min extension step at 72⁰ C. DNA was then prepared using a QIAfilter Plasmid Maxi Kit (QIAGEN Inc., Santa Clarita), and the ER genomic insert was confirmed by dideoxy sequencing [24].

The ER α genomic DNA was then used as a template to generate a series of 5'-deletion fragments. First, another set of PCR primers were designed with an added Kpn site to facilitate subcloning into the luciferase reporter vector pGL3-Basic (Promega, Madison, WI). The 5' primers were ERP-0.245 (5'-⁻²⁴⁵GCGGTACCCC TTTAGCAGAT CCTCGTGC⁻²¹⁷), ERP-0.735 (5'-⁻⁷³⁵GCGGTACCCC TAATGGGACC AAGTACAG⁻⁷⁰⁸), ERP-2.7 (5'-⁻²⁷⁶⁹GCGGTACCGA TCCATGTGAA CGCCACTGG⁻²⁷⁴⁰), ERP-1.0 (5'-⁻¹⁰⁰⁰GCGGTACCCG AGAAGATCGA GTTGTAGGAC⁻⁹⁷⁰) [14], and ERP-4.1 (5'-⁻⁴¹⁰⁰CCGGTACCGG ATCCTGAAGC AGTA⁻⁴⁰⁷⁷) [17]. All PCR fragments were generated with the same 3' primer, ERP+0.212 (5'-⁺²¹²CGCTCGAGTG CAGACCGTGT CCCC GCAGG⁺¹⁸³), which included an added Xho I site for cloning purposes (see Fig. 1A, A-E respectively). PCR amplification was carried out using the Expand High-Fidelity polymerase (Boehringer Mannheim, Indianapolis, IN), with the same parameters described previously.

To create EcoRI-linker mutations scanning the -235 bp to -192 bp region of the ER α promoter, a modified version of the three-step PCR mutagenesis strategy described by Li and Shapiro [25] was used. These were then subcloned into the Kpn I-Xho I sites of the pGL3-Basic luciferase reporter plasmid. The PCR primers were designed to substitute an EcoRI-linker sequence (underlined below) for the wild-type ER α promoter sequence as well as to include a 5' Kpn I site for subcloning purposes. These primers include: an EcoRI site at -235 (5'-

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-245 ACCTTTAGCA CCGGAATTCC GGCCCCCGCC CC⁻²¹⁴), an EcoRI site at -223 (5'-⁻²⁴⁵ACCTTTAGCA GATCCTCGTG CGCCGGAATT CCGGGGCCGT GAAA⁻²⁰²), an EcoRI site at -211 (5'-⁻²⁴⁵ACCTTTAGCA GATCCTCGTG CGCCCCCGCC CCCTGGAATT CCAACTCAGC CT⁻¹⁹⁴), and an EcoRI site at -203 (5'-⁻²⁴⁵ACCTTTAGCA GATCCTCGTG CGCCCCCGCC CCCTGGCCGT GACCCGGAATT CCGGATCCAG CAGC⁻¹⁸²). Briefly, the PCR reactions (25 μ l) contained 100 ng of the luciferase reporter construct containing the -245 to +212 bp fragment and 5 units Amplitaq DNA polymerase (Perkin-Elmer, Foster City, CA). The first PCR amplification was carried out with 20 pmole of each mutation primer paired with 10 pmole of the GL3-2 downstream vector primer (Promega, Madison, WI). The reactions were carried out at 96 °C for 15 sec, 45 °C for 30 sec, and 72 °C for 3 min for 35 cycles, followed by an additional cycle with a 10-min 72 °C extension. Five μ l of the products from the first round of amplification were then used directly in a second round of asymmetric amplification with the same primers and volumes used in the first round. The second round PCR conditions were 96 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 3 min for 35 cycles with the additional extension cycle. The products from the second round were then gel-purified and 5 μ l used as the template in a third round of amplification using the same conditions as the second round. The products were gel purified, digested with Kpn I and Xho I restriction enzymes, and again gel-purified. All constructs and mutations were confirmed by dideoxy sequencing. Plasmid DNA was again prepared using a QIAfilter Plasmid Maxi Kit.

2.2. Cell Culture and Transfection-

MCF-7C cells were kindly provided by Dr. Powel Brown and maintained in Improved Minimal Essential Medium (IMEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, Mo.) and 6 ng of bovine insulin (Sigma, St. Louis, MO) per ml. All of the other cell lines (T47-D, ZR-75-1, MDA-MB-231, and MDA-MB-435) were obtained from American Tissue Culture Collection (Rockville, MD) and maintained in complete Minimal Essential Medium (MEM) (Gibco/BRL, Gaithersburg, Md.) supplemented with 10% FBS. Cells were incubated at 37 °C in 5% CO₂.

Drosophila Schneider SL2 cells (American Type Culture Collection, Rockville, MD) were maintained in Schneider's *Drosophila* Medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% FBS,

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penicillin/streptomycin at 100 U/ml, and Amphotericin B fungazone (Cellgro, Herndon, VA) at 2.5 μ g/ml. Cells were incubated at room temperature without CO₂.

Transient transfections with the human cell lines were performed two or more times in triplicate wells. Cells were seeded in 6-well cluster plates (Falcon, Franklin Lakes, NJ) at a density of 2×10^5 cells/well 24 hours prior to transfection. For each well, 8 μ l of Lipofectamine (Gibco/BRL, Gaithersburg, MD) were combined with 100 μ l of OptiMEM media (Gibco/BRL), and then added to 0.5 μ g of ER luciferase reporter construct plus 0.1 μ g of pCMV- β -galactosidase (Promega, Madison, WI) to correct for transfection efficiency. Cells were incubated overnight with the DNA/lipofectamine mixture, and then washed in phosphate buffered saline. Fresh growth medium was applied and the cells were incubated for 24 additional hours before harvesting for luciferase determinations. Luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison, WI) based on the method of Rouet et al [26]. Luciferase values were normalized using the β -galactosidase values.

Transient transfection assays with the SL2 cells were carried out as described above with the following modifications: Cells were seeded 24 hours prior to transfection at a concentration of 2×10^5 cells/well. 4.5 μ l of FuGene 6 transfection reagent (Boehringer Mannheim, Indianapolis, IN) was used to transfect 0.5 μ g of the ER luciferase reporter construct containing the -245 to +212 bp fragment, along with 0.5 μ g of vector DNA (pPac) or increasing concentrations of expression plasmids for either Sp1 (pPacSp1 [27], (a kind gift of Dr. Robert Tjian (University of California at Berkeley), or Sp3 (pPacUSp3 [28], generously provided by Dr. Guntram Suske, Philipps-Universitat Marburg, Germany). Both expression plasmids contain the coding sequence of Sp1 or Sp3 inserted downstream of the actin 5C promoter. The plasmid pNull-Renilla (Promega), an expression plasmid for the Renilla luciferase gene void of eukaryote promoter or enhancer sequences, was co-transfected for transfection normalization. Luciferase activity was measured using the Dual Luciferase kit from Promega. Mithramycin A (Sigma, St. Louis, MO), an inhibitor of Sp1 DNA binding [29], was also added 24 hours after transfection in some experiments at concentrations of 5×10^{-8} M to 5×10^{-6} M.

For Mithramycin A studies, MCF-7 cells were seeded 24 hours prior to transfection at a concentration of 1×10^5 cells/well. Again, 4.5 μ l of FuGene 6 transfection reagent (Boehringer Mannheim) was used to transfect 0.5 μ g of the ER α promoter luciferase reporter construct containing the -245 to +212 bp fragment. The plasmid pNull-Renilla (Promega) was co-transfected for transfection normalization. Mithramycin A (Sigma) was added 24 hours

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after transfection at concentrations of 10^{-9} M, 10^{-8} M, and 10^{-7} M. Cells were harvested 18 hours after treatment and luciferase activity was measured using the Dual Luciferase kit from Promega.

2.3. Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were carried out as previously published [30], with a few modifications. A probe was generated using PCR as previously described with ER α promoter primers 5'-⁻²⁴⁵ACCTTAGCAG ATCCTCGT⁻²²⁵ and 5'-⁻¹⁸²GCTGCTGGAT AGAGGCTGA⁻²⁰⁰. The PCR fragment was then ³²P-end-labeled using T4 kinase as previously described [30]. The radiolabeled probe (20,000 cpm) was added to 30 μ g of high salt cellular extract [30] from MCF-7 cells, in a final reaction buffer of 10 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl₂, and 10 % glycerol, along with 3 μ g of poly dIdC in a final reaction volume of 20 μ l. Reactions were incubated for 30 minutes at room temperature. Oligonucleotides for competitive binding studies were preincubated with the MCF-7 extract for 5 min at room temperature before the addition of radiolabeled probe. The sequence for the consensus Sp1 oligonucleotide was 5'-ATT CGA TCA GGG CGG GGC GAG C -3'. For supershift assays, cell extracts were preincubated with 2 μ g of either Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), or AP2 (C-18) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min on ice. The reactions were then separated on a 5% polyacrylamide gel with 0.5X TBE buffer electrophoresed for 5 hours at 150 volts at 4 °C. The gel was dried and exposed to film (Kodak X-OMAT).

2.4. Western Blot Analysis

MCF-7 cells were seeded in 6-well plates at a density of 2×10^5 cells per well 24 hours prior to treatment. Cells were then treated with Mithramycin A (Sigma, St. Louis, MO) for 18 hours, harvested in a 5% SDS solution, and 10 μ g of protein extract were resolved on a polyacrylamide gel as previously described [31]. These were then transferred from the gel to nitrocellulose membranes and subjected to immunodetection [31] with the ER-specific 6F11 monoclonal antibody (Novocastra Ltd., England), the Sp1 and Sp3 antibodies, or tubulin (Chemicon, Temecula, CA) for a loading control, and the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL). Densitometry analysis was done using NIH Image 1.62 software.

2.5. Statistical Analysis-Promoter Activity

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For each of 5 human breast cancer cell lines, luciferase activity from triplicate wells was used to measure promoter activity of 5 promoter fragments and vector alone. Each experiment was completely replicated. Luciferase data were log-transformed prior to analysis, as indicated by a Box-Cox analysis [32]. Two-way analysis of variance, with promoter construct as one factor and experiment as the other, was used to evaluate differences among constructs after adjusting for experiment-to-experiment differences. Pairwise comparisons of least-squares estimated means for each construct were used to determine which constructs had similar activities. P-values were adjusted for multiple comparisons by the Sidak method, which is similar to but less excessively conservative than the Bonferroni method [33]. For purposes of presentations, means and 95% confidence intervals were back-transformed to give estimates of average and 95% confidence intervals of fold-increases in relative luciferase activity.

Linker-Scanner Mutated Fragments: Luciferase activity from triplicate wells was analyzed using one-way analysis of variance. Box-Cox analysis did not indicate a need for transformation. Pairwise comparisons were used to determine which fragments had similar activities. P-values were adjusted for multiple comparisons by the Sidak method. Analyses were performed using SAS (Version 6.12, SAS Institute, Cary, NC).

All other statistical analysis were performed using the Student T test, comparing the difference between control and experimental values.

3. Results

The Majority Of ER α Promoter Activity Resides Within The Most Proximal 245 Bases Of 5'-Flanking DNA-To identify regulatory regions involved in ER promoter transcriptional activity, five overlapping ER promoter deletion constructs, -245 bp to +212 bp, -735 bp to +212 bp, -1000 bp to +212 bp, -2769 bp to +212 bp, and -4100 bp to +212 bp (Fig.1, fragments A-E respectively), all relative to the first transcriptional start site (Fig. 1, P1), were subcloned into the luciferase reporter vector pGL3-Basic. [INSERT FIG 1 HERE] Since we have previously shown that the region between -245 bp to -9 bp contains low basal activity [34], we designed our smallest fragment (Fig. 1, fragment A) to include this region, as well as the two binding sites for AP2 γ identified by deConinck, *et al* [18], within the 5' untranslated region. We extended the next fragment (Fig. 1, fragment B) out to -735 bp to include a 1/2 estrogen response element (ERE) at -420 bp [35]. Fragment C was then extended 5' to include two other 1/2 EREs located at -860 and -888 bp. Fragments D and E were designed to include the P0 transcription start [13]; fragment E also contains the ER-EH0 enhancer [17].

These five constructs were transiently transfected into a panel of breast cancer cell lines that included the ER-positive MCF-7, T47D, and ZR75-1, as well as the ER-negative MDA-MB-231 and MDA-MB-435 (Fig 2) cell lines. [INSERT FIG 2 HERE] Data is shown as fold increase in luciferase units over vector control values. Fragment A exhibited high levels of activity in all the cell lines (Fig. 2A, panel A), displaying a 7 to 20-fold increase compared to vector alone, depending on the cell line. This supports our earlier observation [34] that this region contains important regulatory elements necessary for ER α transcription

The activity profiles of the ER-positive cell lines were essentially the same. The activity of fragments B and C did not significantly change in MCF-7, T47D, or ZR-75-1 cells, but was slightly increased (2-fold) relative to fragment A in ZR-75-1 cells. The activity of fragment D significantly decreased ($p < 0.04$) in all of the ER-positive cell lines relative to the activity seen with the A fragment, suggesting the presence of negative regulatory elements between -1000 to -2769 bp. In contrast, the activity of fragment E increased ($p < 0.003$) in all three ER-positive cell lines, consistent with the data of Tang *et al* [17] who has previously characterized the ER-EH0 enhancer element within this region of the ER α promoter.

The activity profiles of the longer ER α promoter fragments were different in the ER-negative breast cancer cell lines compared the ER-positive cells. The activity of fragments B-E was significantly reduced compared to fragment A in the two ER-negative cell lines ($p < 0.005$) (Fig. 2A). Since fragments B-E all have the region between -245 to -735 bp, it is possible that this region of the ER promoter contains potential negative regulatory elements

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that are utilized in ER-negative cells, but not ER-positive cells for reasons that we do not yet know. This will be pursued in a future study.

Because the majority of ER α promoter activity in all of the breast cancer cell lines resided within the first -245 bp, we next focused our attention to this region of the promoter with the goal of identifying those factors critical for regulating its transcription. To do this we generated a promoter construct deleting the region between -245 and -200 bp, because an initial search for potential transcription factor binding sites [36] identified a number of sites within this region. This promoter deletion construct was then examined in MCF-7 cells using transient transfection analysis (Fig. 2B). Because the activity of fragment A in MCF-7 cells was representative of all the ER-positive cell lines, we chose this cell line for the next series of experiments. The -245 fragment (Fig. 2B, second bar) exhibited a 10-20 fold increase in activity, depending on the experiment, over the control vector alone. The removal of the -245 to 200 bp region (the -200 fragment, Fig. 2B, third bar) invariably resulted in the loss of all promoter transcriptional activity. These results suggest that elements critical for transcriptional activity of the ER α promoter lie within the -245 and -200 bp region; this region thus contains the ER minimal promoter.

Multiple Elements Are Required For Full ER α Promoter Activity-To more precisely define the functional elements within the ER α minimal promoter that confer transcriptional activation, eight to twelve bp EcoRI linker-scanner mutations directed at known regulatory elements, such as the non-consensus E-box at -231 to -226 bp, the GC box at -223 to -214 bp, and a C/A rich box at -203 to -192 bp (Fig. 3) were introduced into the ER α promoter reporter fragment A (Fig. 4, starred regions). [INSERT FIG. 3 AND 4 HERE] These elements are potential binding sites for basic helix-loop-helix transcription factors and Sp1 transcription factor family members, respectively [37-40]. The linker-scanner mutation reporter constructs were then transfected into MCF-7 cells and the results expressed as fold activity compared to vector control (Fig. 4). Mutation of the region between -235 and -224 bp (Fig. 4, Fragment A1) resulted in a 37% loss of promoter activity. Even more significant activity losses of 63% and 81% respectively, were seen with fragments A2 and A4. In comparison, a negligible decrease in activity was seen when the region between bases -211 and -204 was mutated (Fig. 4, Fragment A3). These results suggest that there are critical elements contained within Fragments A2 (the region between -223 and -212 bp) and A4 (-203 to -192 bp). Furthermore, the significant reduction also seen when the region in Fragment A1 is mutated suggests that multiple elements, and their cognate binding factors, may cooperate for full ER α transcription activity of the minimal promoter.

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Sp1 and Sp3 Interact with the ER Proximal Promoter Region-To determine which transcription factors might be binding to the critical elements within fragment A, a radiolabeled oligonucleotide probe spanning the ER α promoter region from -245 to -182 bp (Fig. 3) was used in an EMSA to detect potential binding factors. Seven specific DNA-protein complexes were detected with MCF-7 protein extracts (Fig. 5, lane A, arrows labeled 1 through 7). [INSERT FIG. 5 HERE] All seven of these complexes were specifically competed by a 25-fold excess of unlabeled probe (cold competitor, lane B), or an excess of an oligonucleotide containing a consensus binding site for Sp1 family members (lane C). The specificity of these competition assays was also confirmed using an oligonucleotide with a mutated Sp1 binding site (data not shown).

The identity of the protein-DNA complexes was investigated with supershift analysis with antibodies to Sp1 and Sp3. It appears that most of the seven complexes indeed contain Sp1 and/or Sp3 (lanes D, E, and H). The most demonstrable difference in the supershifted complexes was seen with complex 7. This complex clearly contains Sp3, but not Sp1 (compare lanes D and E). Incubation of the MCF-7 extract with an antibody to either Sp4 (lane F) or AP2 (lane G) prior to addition of probe had no effect on any of these complexes. However, when both Sp1 and Sp3 antibodies are preincubated with the extract, all seven of the complex bands were affected (lane H). These data suggest that both Sp1 and Sp3 bind to the minimal ER promoter region.

Sp1 and Sp3 are Able To Directly Transactivate the ER Minimal Promoter-In order to determine whether Sp1 and Sp3 are capable of transactivating the ER α minimal promoter (ER promoter reporter fragment A), we performed a series of transient transfections in *Drosophila* SL2 cells. These cells do not express any of the Sp1 family members. In this experiment, we also treated the transiently transfected cells with the Sp1 DNA-binding inhibitor Mithramycin A (29) to determine its effect on exogenous Sp1 and Sp3 activity. As shown in Fig. 6, both Sp1 and Sp3 were capable of transactivating the ER α minimal promoter in a dose-dependent manner. [INSERT FIG 6 HERE] Exogenous expression of Sp1 was able to increase activity of the minimal promoter fragment 3 to 5-fold (Fig. 6, lanes 2 and 3), and expression of Sp3 increased promoter activity 4 to 6-fold (Fig. 6, lanes 8 and 9). Furthermore, this transactivation was inhibited in a dose-dependent manner by increasing concentrations of Mithramycin A. Concentrations of drug at 5×10^{-8} M slightly inhibited promoter activity in Sp1 transfected cells (Fig. 6, lane 4), but significantly affected transactivation of the minimal promoter in Sp3 transfected cells ($p \leq 0.001$, Fig 6, lane 10). This effect was further enhanced with increasing concentrations of drug in both Sp1 and Sp3 transfected cells.

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These results show that Sp1 and Sp3 are independently capable of transactivating the ER α minimal promoter in *Drosophila* SL2 cells, and that this transactivation is due to binding of these proteins to the promoter, since this binding could be inhibited by Mithramycin A.

The significance of binding by Sp1 and/or Sp3 to the ER α minimal promoter for transcriptional activity was confirmed in data shown in Fig. 7. [INSERT FIG 7 HERE] MCF-7 cells transiently transfected with the ER minimal promoter fragment A, and treated with increasing concentrations of Mithramycin A, demonstrated a dose-dependent loss of promoter activity (Fig. 7, panel A, $p \leq 0.006$) as well as a decrease in endogenous ER α protein levels (Fig. 7, panel B). ER α promoter activity was decreased approximately 60% in cells treated with 10^{-9} M drug, 70% in cells treated with 10^{-8} M drug, and almost 80% in cells treated with 10^{-7} M drug, as compared to the promoter activity in cells treated with ethanol alone. The effects on ER α promoter activity were correlated with the effects on ER α expression levels (Fig. 7, panel B). ER α protein levels decreased 20% in cells treated with 10^{-9} M Mithramycin A as compared to control. This decrease in expression was more dramatic in cells treated with 10^{-8} M drug (62%). In cells treated with 10^{-7} M drug, expression of ER α was below detectable levels. The levels of Sp1 and Sp3 were unaffected by Mithramycin A (Tubulin levels were used as a control for loading in the Western blot, Fig. 7, panel B). Taken together with the SL2 transactivation data, these results demonstrate an essential role for Sp1 family members in transcription of the ER α gene in breast cancer cells.

4. Discussion

Exploring the factors influencing ER α expression has been the focus of a number of studies [12-14,16-18,34,35]. These studies provide strong evidence that ER α expression in breast cancer is regulated at both the transcriptional as well as the translational level. To date, few definitive transcriptional regulatory regions have been identified within the ER α gene, and except for the ER itself [34,35], and possibly AP2 γ [20], no transcription factor has been identified which can directly transactivate the ER α promoter. A better understanding of the specific factors that can influence ER α expression in breast cancer cells is critical for future strategies attempting to regulate its expression.

In this study, we first analyzed the activity of fragments spanning +212 to -4100 bp of the ER α promoter and 5' untranslated region in a panel of breast cancer cell lines, thus identifying regions of the promoter to focus upon for closer study. Previous literature suggested regions with potential regulatory activity [17,18,34,35], upon which we based a strategy for construction of reporters with ER α promoter deletions. The results of these deletion experiments suggest that while there appears to be enhancer activity located between nucleotides -2769 and -4100, as was originally proposed by Tang *et al.* [17], the majority of ER α promoter activity lies within the first 245 bp of the 5'-flanking region of the ER gene. We also found a statistically significant loss of activity ($p < 0.005$) in ER-negative cells with the longer ER promoter fragments, which was not evident in the ER-positive cells. Thus the overall profiles of ER-positive and ER-negative cells were distinct. This might indicate the presence of a negative regulator within the -1000 to -2769 bp region of the promoter. This observation will be a focus for future studies.

We chose to first identify those factors that were essential for ER α expression. Since the majority of ER α promoter activity was located within the first 245 bp of the proximal promoter, we more closely analyzed this region. Our deletion studies indicated that this region of the promoter contained elements critical for transcriptional activity, thus defining this region as the minimal ER promoter. We then used linker-scanner analysis of this smallest defined minimal promoter region to identify potential regulatory regions. We demonstrated that two GC/CA rich boxes, as well as a non-consensus E box, were essential for full transcriptional activity of the ER α minimal promoter. GC/CA rich boxes classically bind members of the Sp1 family of transcription factors [37,39,41], and these factors have proven critical for full promoter activity of many genes. In this study we found that both Sp1 and Sp3 can bind to the minimal promoter region. In addition, these two proteins significantly transactivated the minimal promoter when exogenously expressed in *Drosophila* SL2 cells. Sp3 was once believed to be solely a

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repressor factor [28,42], but recently an activation role for Sp3 has also been demonstrated [41,43]. We conclude that one, if not both, of these two transcription factors are important for transcriptional regulation of ER α .

One potential mechanism how Sp1 and Sp3 affect ER α expression could involve a direct protein:protein interaction between Sp1 and ER α . We have previously shown that one mechanism by which ER α autoregulates this promoter region is by a protein:protein interaction with an as yet unidentified factor [34]. There is also precedence for ER-Sp1 interactions for transactivation of other promoters [44-47]. We are currently investigating the possibility of such an interaction on the ER α promoter.

Our results also suggest that Sp1 and Sp3 do not function alone in increasing ER α expression. Many studies indicate that Sp1 can participate in multi-protein complexes with diverse interacting partners to regulate transcription of certain genes [48,49]. Our linker-scanner analyses suggest that regions bordering the putative binding sites for the Sp1 transcription factors are also important for full promoter activity. The formation of a multi-protein, DNA-binding complex containing Sp1 and Sp3, as well as other as yet unidentified factors, is consistent with our detection of seven protein/DNA complex bands. Identifying the additional components within these complexes could prove critical for understanding the precise mechanism involved in regulating ER α expression.

In summary, we have demonstrated that the majority of ER α promoter activity in breast cancer cell lines lies within a minimal promoter located in the first 245 bp of the 5'-flanking region of the ER α gene. We have localized elements required for full ER α transcriptional activity to at least two potential binding sites for the Sp1 family of transcription factors, and have demonstrated that both Sp1 and Sp3 present in cellular extracts from breast cancer cells are capable of binding to this region. We have also shown that both of these factors are able to transactivate the ER α minimal promoter in *Drosophila* SL2 cells, and that interference with Sp1/Sp3 DNA binding results in a loss of ER α promoter activity as well as ER α protein expression in MCF-7 cells. These data strongly suggest an important role for these two proteins in transcription of the ER α gene. We anticipate that the results of this study will be a basis for the identification of other factors involved in the regulation of ER α transcription.

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References

1. J.C. Allegra, M.E. Lippman, L. Green, A. Barlock, R. Simon, E.B. Thompson, K.K. Huff and W. Griffin, Estrogen receptor values in patients with benign breast disease, *Cancer* 44 (1979) 228-31.
2. G.J. Netto, J.H. Cheek, N.Y. Zachariah, J.C. Romer, C.F. Lee, R. Schoenfeld and Z.H. Chakmakjian, Steroid receptors in benign mastectomy tissue, *Am J Clin Pathol* 94 (1990) 14-7.
3. T. van Agthoven, M. Timmermans, J.A. Foekens, L.C. Dorssers and S.C. Henzen-Logmans, Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry, *Am J Pathol* 144 (1994) 1238-46.
4. S.A. Khan, M.A. Rogers, J.A. Obando and A. Tamsen, Estrogen receptor expression of benign breast epithelium and its association with breast cancer, *Cancer Res* 54 (1994) 993-7.
5. S.A. Khan, M.A. Rogers, K.K. Khurana, M.M. Meguid and P.J. Numann, Estrogen receptor expression in benign breast epithelium and breast cancer risk [see comments], *J Natl Cancer Inst* 90 (1998) 37-42.
6. M.A. Fanelli, L.M. Vargas-Roig, F.E. Gago, O. Tello, R. Lucero De Angelis and D.R. Ciocca, Estrogen receptors, progesterone receptors, and cell proliferation in human breast cancer, *Breast Cancer Res Treat* 37 (1996) 217-28.
7. B.E. Henderson and L. Bernstein, Endogenous and Exogenous Hormonal Factors, J.R. Harris, M.E. Lippman, M. Morrow and S. Hellman (Eds), 185-200, Lippincott - Raven, Philadelphia - New York (1996).
8. P.J. Barrett-Lee, M.T. Travers, R.A. McClelland, Y. Luqmani and R.C. Coombes, Characterization of estrogen receptor messenger RNA in human breast cancer, *Cancer Res* 47 (1987) 6653-9.
9. J.A. Henry, S. Nicholson, J.R. Farndon, B.R. Westley and F.E. May, Measurement of oestrogen receptor mRNA levels in human breast tumours, *Br J Cancer* 58 (1988) 600-5.
10. D. Ricketts, L. Turnbull, G. Ryall, R. Bakhshi, N.S. Rawson, J.C. Gazet, C. Nolan and R.C. Coombes, Estrogen and progesterone receptors in the normal female breast, *Cancer Res* 51 (1991) 1817-22.
11. M.C. Rio, J.P. Bellocq, B. Gairard, U.B. Rasmussen, A. Krust, C. Koehl, H. Calderoli, V. Schiff, R. Renaud and P. Chambon, Specific expression of the pS2 gene in subclasses of breast cancers in comparison

Influence of Sp1 on ER α Gene Transcription

- with expression of the estrogen and progesterone receptors and the oncogene ERBB2, *Proc Natl Acad Sci U S A* 84 (1987) 9243-7.
12. R.J. Weigel and E.C. deConinck, Transcriptional control of estrogen receptor in estrogen receptor- negative breast carcinoma, *Cancer Res* 53 (1993) 3472-4.
 13. R. Piva and L. Del Senno, Analysis of a DNA sequence upstream of the human estrogen receptor gene, *Ann N Y Acad Sci* 684 (1993) 235-8.
 14. S. Green, P. Walter, V. Kumar, A. Krust, J.M. Bornert, P. Argos and P. Chambon, Human oestrogen receptor cDNA: sequence, expression and homology to v- erb-A, *Nature* 320 (1986) 134-9.
 15. D.A. Thompson, L.A. McPherson, C. Carmeci, E.C. deConinck and R.J. Weigel, Identification of two estrogen receptor transcripts with novel 5' exons isolated from a MCF7 cDNA library, *J Steroid Biochem Mol Biol* 62 (1997) 143-53.
 16. K. Grandien, Determination of transcription start sites in the human estrogen receptor gene and identification of a novel, tissue-specific, estrogen receptor-mRNA isoform, *Mol Cell Endocrinol* 116 (1996) 207-12.
 17. Z. Tang, I. Treilleux and M. Brown, A transcriptional enhancer required for the differential expression of the human estrogen receptor in breast cancers, *Mol Cell Biol* 17 (1997) 1274-80.
 18. E.C. deConinck, L.A. McPherson and R.J. Weigel, Transcriptional regulation of estrogen receptor in breast carcinomas, *Mol Cell Biol* 15 (1995) 2191-6.
 19. L.A. McPherson, V.R. Baichwal and R.J. Weigel, Identification of ERF-1 as a member of the AP2 transcription factor family, *Proc Natl Acad Sci U S A* 94 (1997) 4342-7.
 20. L.A. McPherson and R.J. Weigel, AP2alpha and AP2gamma: a comparison of binding site specificity and trans-activation of the estrogen receptor promoter and single site promoter constructs, *Nucleic Acids Res* 27 (1999) 4040-4049.
 21. B.C. Turner, J. Zhang, A.A. Gumbs, M.G. Maher, L. Kaplan, D. Carter, P.M. Glazer, H.C. Hurst, B.G. Haffty and T. Williams, Expression of AP-2 transcription factors in human breast cancer correlates with the regulation of multiple growth factor signalling pathways, *Cancer Res* 58 (1998) 5466-72.
 22. U.J. Kim, B.W. Birren, T. Slepak, V. Mancino, C. Boysen, H.L. Kang, M.I. Simon and H. Shizuya, Construction and characterization of a human bacterial artificial chromosome library, *Genomics* 34 (1996) 213-8.

Influence of Sp1 on ER α Gene Transcription

23. J.S. Kovach, R.M. McGovern, J.D. Cassady, S.K. Swanson, L.E. Wold, B. Vogelstein and S.S. Sommer, Direct sequencing from touch preparations of human carcinomas: analysis of p53 mutations in breast carcinomas [see comments], *J Natl Cancer Inst* 83 (1991) 1004-9.
24. F. Sanger, S. Nicklen and A.R. Coulson, DNA sequencing with chain-terminating inhibitors, *Proc Natl Acad Sci U S A* 74 (1977) 5463-7.
25. X.M. Li and L.J. Shapiro, Three-step PCR mutagenesis for 'linker scanning', *Nucleic Acids Res* 21 (1993) 3745-8.
26. P. Rouet, G. Raguenez and J.P. Salier, Optimized assays for quantifying transient expressions of co-transfected beta-galactosidase and CAT reporter genes, *Biotechniques* 13 (1992) 700-1.
27. A.J. Courey and R. Tjian, Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif, *Cell* 55 (1988) 887-98.
28. G. Hagen, S. Muller, M. Beato and G. Suske, Sp1-mediated transcriptional activation is repressed by Sp3, *Embo J* 13 (1994) 3843-51.
29. R. Ray, R.C. Snyder, S. Thomas, C.A. Koller and D.M. Miller, Mithramycin blocks protein binding and function of the SV40 early promoter, *J Clin Invest* 83 (1989) 2003-7.
30. S.A. Fuqua, M.G. Benedix, S. Krieg, C.N. Weng, G.C. Chamness and S. Oesterreich, Constitutive overexpression of the 27,000 dalton heat shock protein in late passage human breast cancer cells, *Breast Cancer Res Treat* 32 (1994) 177-86.
31. A.K. Tandon, G.M. Clark, G.C. Chamness, A. Ullrich and W.L. McGuire, HER-2/neu oncogene protein and prognosis in breast cancer, *J Clin Oncol* 7 (1989) 1120-8.
32. G. Box and D. Cox, Analysis of Transformation, *Journal of the Royal Statisticians Society* 26 (1964) 211-252.
33. P. Westfall and S. Young, Resampling Based Multiple Testing, John Wiley & Sons, Inc., New York.
34. C.G. Castles, S. Oesterreich, R. Hansen and S.A. Fuqua, Auto-regulation of the estrogen receptor promoter, *J Steroid Biochem Mol Biol* 62 (1997) 155-63.
35. Treilleux, N. Peloux, M. Brown and A. Sergeant, Human estrogen receptor (ER) gene promoter-P1: estradiol-independent activity and estradiol inducibility in ER⁺ and ER⁻ cells, *Mol Endocrinol* 11 (1997) 1319-31.
36. Y. Akiyama, *TFSEARCH* (1.3 Ed), Vol. 1997, Kyoto University, Japan (1995).

Influence of Sp1 on ER α Gene Transcription

37. K.A. Jones and R. Tjian, Sp1 binds to promoter sequences and activates herpes simplex virus 'immediate-early' gene transcription in vitro, *Nature* 317 (1985) 179-82.
38. C.B. Bigger, E.A. Casanova and P.D. Gardner, Transcriptional regulation of neuronal nicotinic acetylcholine receptor genes. Functional interactions between Sp1 and the rat beta4 subunit gene promoter, *J Biol Chem* 271 (1996) 32842-8.
39. K.D. Fischer, A. Haese and J. Nowock, Cooperation of GATA-1 and Sp1 can result in synergistic transcriptional activation or interference, *J Biol Chem* 268 (1993) 23915-23.
40. L. Vindevoghel, K.Y. Chung, A. Davis, D. Kouba, S. Kivirikko, H. Alder, J. Uitto and A. Mauviel, A GT-rich sequence binding the transcription factor Sp1 is crucial for high expression of the human type VII collagen gene (COL7A1) in fibroblasts and keratinocytes, *J Biol Chem* 272 (1997) 10196-204.
41. C.B. Bigger, I.N. Melnikova and P.D. Gardner, Sp1 and Sp3 regulate expression of the neuronal nicotinic acetylcholine receptor beta4 subunit gene, *J Biol Chem* 272 (1997) 25976-82.
42. P. De Luca, B. Majello and L. Lania, Sp3 represses transcription when tethered to promoter DNA or targeted to promoter proximal RNA, *J Biol Chem* 271 (1996) 8533-6.
43. B. Majello, P. De Luca and L. Lania, Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains, *J Biol Chem* 272 (1997) 4021-6.
44. W. Porter, B. Saville, D. Hoivik and S. Safe, Functional synergy between the transcription factor Sp1 and the estrogen receptor, *Mol Endocrinol* 11 (1997) 1569-80.
45. V. Krishnan, X. Wang and S. Safe, Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells, *J Biol Chem* 269 (1994) 15912-7.
46. R. Duan, W. Porter and S. Safe, Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation, *Endocrinology* 139 (1998) 1981-90.
47. A. Scholz, M. Truss and M. Beato, Hormone-induced Recruitment of Sp1 Mediates Estrogen Activation of the Rabbit Uteroglobin Gene in Endometrial Epithelium, *J Biol Chem* 273 (1998) 4360-4366.
48. S. Ryu, S. Zhou, A.G. Ladurner and R. Tjian, The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1, *Nature* 397 (1999) 446-50.
49. J.T. Schanke, M. Durning, K.J. Johnson, L.K. Bennett and T.G. Golos, SP1/SP3-binding sites and adjacent elements contribute to basal and cyclic adenosine 3',5'-monophosphate-stimulated transcriptional activation of the rhesus growth hormone-variant gene in trophoblasts, *Mol Endocrinol* 12 (1998) 405-17.

Influence of Sp1 on ER α Gene Transcription

50. B.L. Kreider, R. Benezra, G. Rovera and T. Kadesch, Inhibition of myeloid differentiation by the helix-loop-helix protein Id, Science 255 (1992) 1700-2.

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FIGURE LEGENDS

Fig. 1. Construction of deletion fragments of the ER α gene promoter. ER α promoter fragments spanning from (A)-245 to +212 bp, (B)- 735 to +212 bp, (C)-1000 to +212 bp, (D)-2769 to +212 bp, and (E)-4100 to +212 bp. Fragments were designed to include the P1 transcriptional start site as well as the putative binding sites for AP2 γ (fragment A), one γ -ERE (fragment B), two additional γ -EREs (fragment C), the secondary transcriptional start site P0 (fragment D), and the ER-EH0 enhancer (fragment E). All the fragments include the ATG at +233 bp. Fragment coordinates are expressed relative to the primary transcription start site.

Fig 2. Promoter activity of the ER α 5'-flanking region. *A*, Constructs described in Fig. 1 were transiently transfected with pCMV- β gal (to correct for transfection efficiency) into a panel of ER α -positive (MCF-7, T47D, and ZR75-1) or ER α -negative (MDA-MB-231 and MDA-MB-435) breast cancer cells. Shown are the geometric mean relative fold luciferase (RFL) units over control, and 95% confidence intervals, summarizing two experiments each performed in triplicate. *B*, ER α promoter deletion fragment A -245/+212 and -200/+212 were transiently transfected with pCMV- β gal into MCF-7 cells. Promoter activity was expressed as corrected fold luciferase activity relative to vector alone and was representative of two independent experiments performed in triplicate wells.

Fig. 3. Schematic representation of the ER α promoter region between nucleotides -245 and -182 bp. Schematic diagram of the potential protein-binding sites located within the ER α minimal promoter region. The non-consensus E box (CTCGTG) located between nucleotides -231 and -226 deviates from the consensus E box at one nucleotide (C \overline{A} NTG), and potentially binds members of the bHLH family of transcription factors [50]. The GC box located between nucleotides -223 and -214 and the CA rich box located between nucleotides -203 and -192 are potential binding sites for the Sp1 family of transcription factors.

Fig. 4. Identification of important regulatory elements in the ER α promoter by linker-scanner mutagenesis. Luciferase reporter constructs containing the wild type ER α minimal promoter fragment A, or linker-scanner mutations of this fragment, were transfected with pCMV- β gal into MCF-7 cells. *Left panel*, schematic representation of the ER α promoter fragments and the potential transcription elements and the linker-scanner mutations generated (star) between nucleotides -235 and -224 (B), nucleotides -223 and -212 (C), nucleotides -211

Influence of Sp1 on ER α Gene Transcription

and -204 (D), and nucleotides -203 and -192 (E). *Right panel*, promoter activity is expressed as relative fold luciferase units over vector control and is representative of two independent experiments done in triplicate.

Fig. 5. Sp1 and Sp3 interact with the -245 to -182 bp region of the ER α promoter. EMSA were performed using MCF-7 extracts. An end-labeled (20,000 cpm) oligonucleotide which spanned the ER promoter from -245 bp to -182 bp (Fig. 3) was incubated with 10 μ g of extract alone (*lane A*), or in the presence of a 25-fold molar excess of cold oligonucleotide (*lane B*), a 25-fold molar excess of a cold oligonucleotide containing an Sp1 consensus binding site (*lane C*), Sp1 antibody (Ab) (*lane D*), Sp3 Ab (*lane E*), Sp4 Ab (*lane F*), AP2 Ab (*lane G*), or both Sp1 and Sp3 Abs (*lane H*). Complexes are indicated by *numbered arrows* and supershifted complexes indicated by *SS*.

Fig. 6. Sp1 transcription factors transactivate the ER α minimal promoter in *Drosophila* cells. *Drosophila* SL2 cells were co-transfected with the ER α minimal promoter luciferase reporter construct and the pPac control vector or increasing concentrations (0.1-0.5 μ g) of either an Sp1 or Sp3 expression vector. Transfected cells were also treated with increasing concentrations of an Sp1 DNA binding inhibitor, Mithramycin A, at concentrations ranging from 5×10^{-8} M to 5×10^{-6} M. Luciferase values were normalized to Renilla luciferase expression. Fold induction was calculated relative to the normalized luciferase activity obtained by transfecting the minimal promoter reporter with the pPac vector alone, and was representative of two independent experiments done in triplicate. # $p \leq 0.001$ compared to control, * $p \leq 0.001$ compared to 0.5 μ g untreated.

Fig. 7. Inhibition of Sp1 DNA binding results in a loss of both ER promoter activity and ER α protein expression in MCF-7 cells. *A*, MCF-7 cells were transiently transfected with the ER α minimal promoter luciferase reporter construct (Fig 1A, A), and were treated with increasing concentrations of an Sp1 DNA binding inhibitor, Mithramycin A, at concentrations ranging from 10^{-9} to 10^{-7} M. Luciferase values were normalized to Renilla luciferase expression. Fold induction was calculated relative to the normalized luciferase activity obtained from the untreated control cells. *B*, MCF-7 cells were treated for 18 hours with concentrations of 0, 5×10^{-9} , 5×10^{-8} , or 5×10^{-7} Mithramycin A and measured for Sp1, Sp3, ER α expression by Western blot analysis. Tubulin staining was used as a control for protein loading.

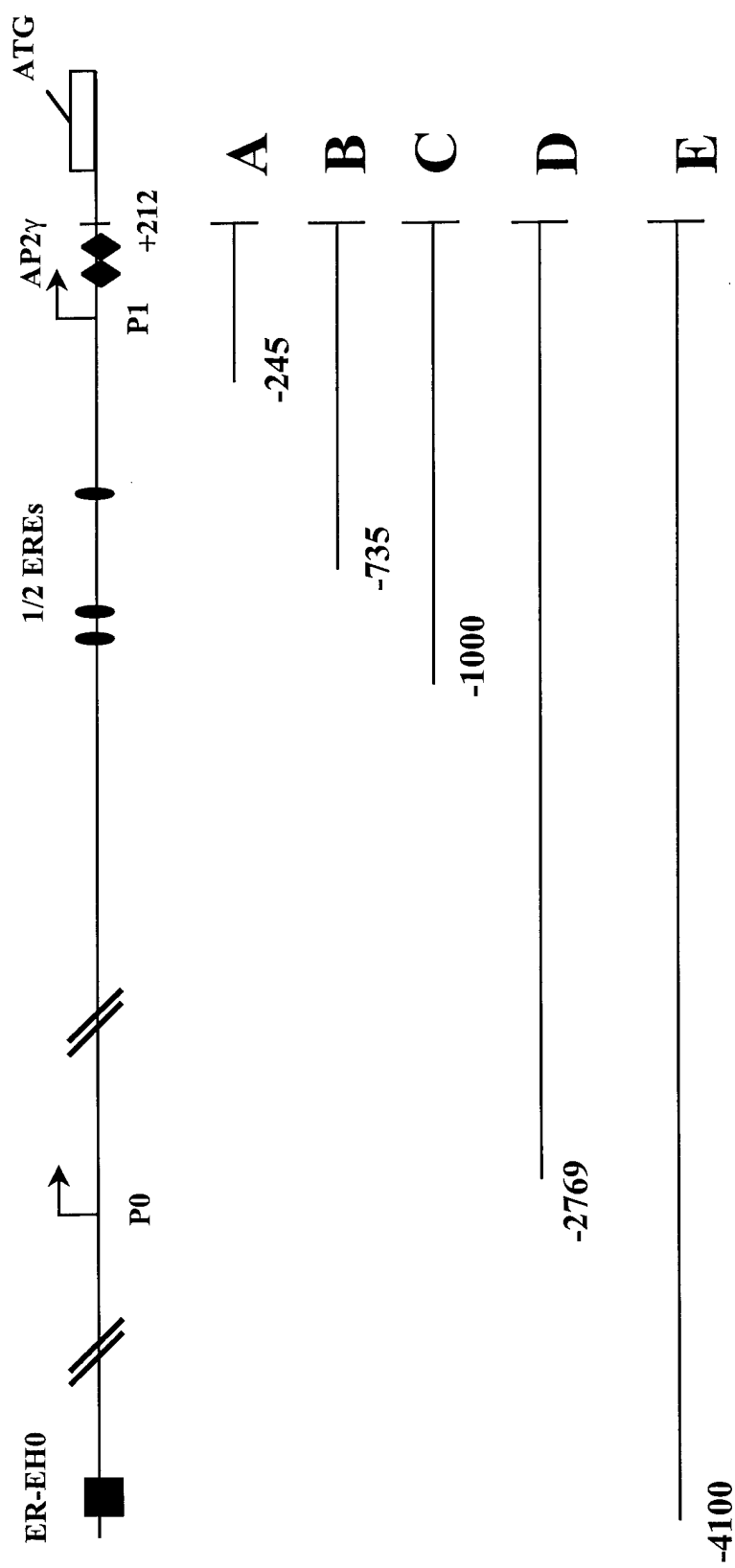


Fig. 1

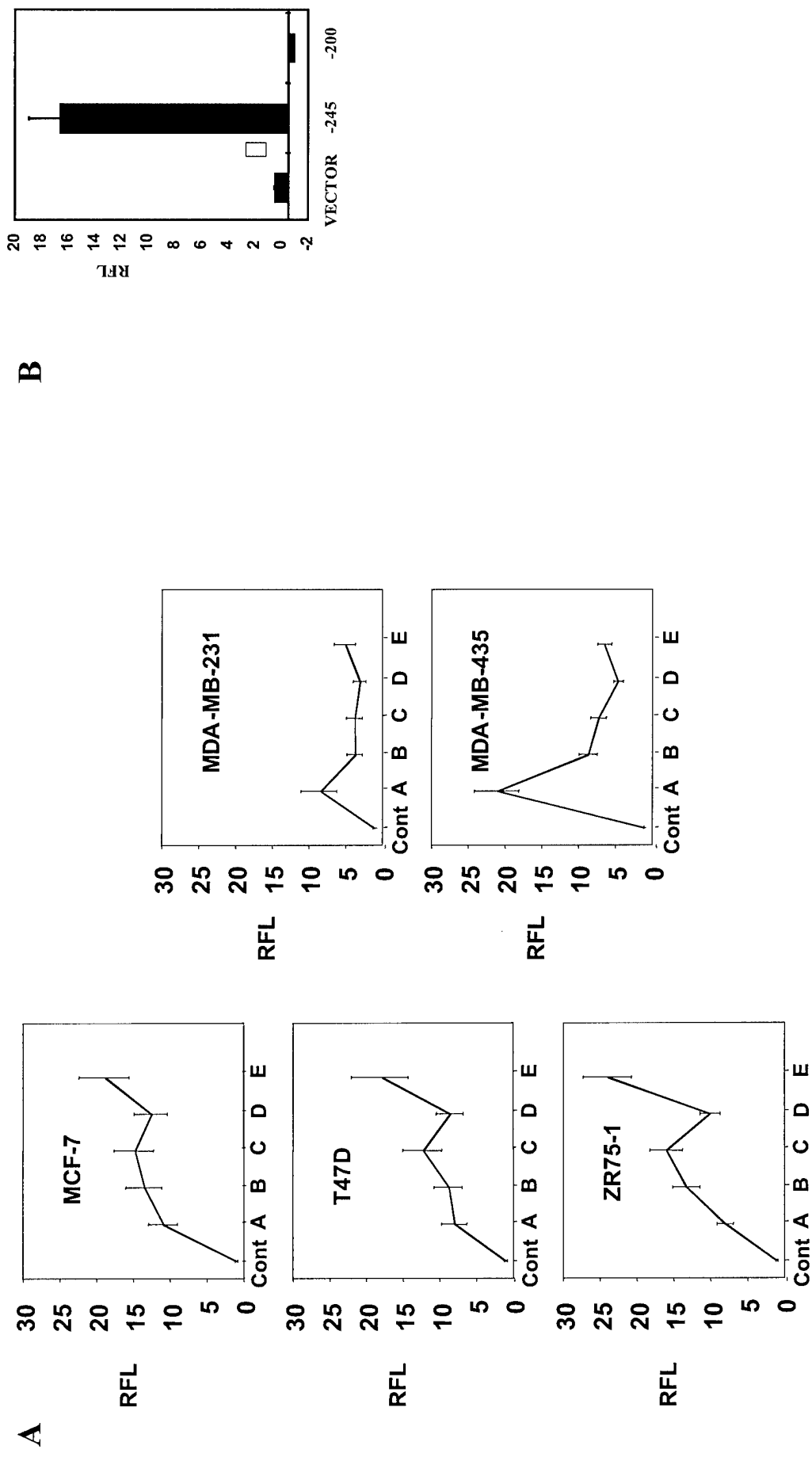


Fig. 2

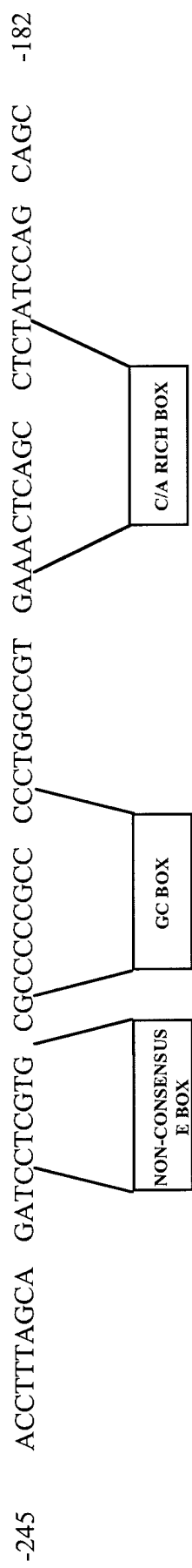


Fig. 3

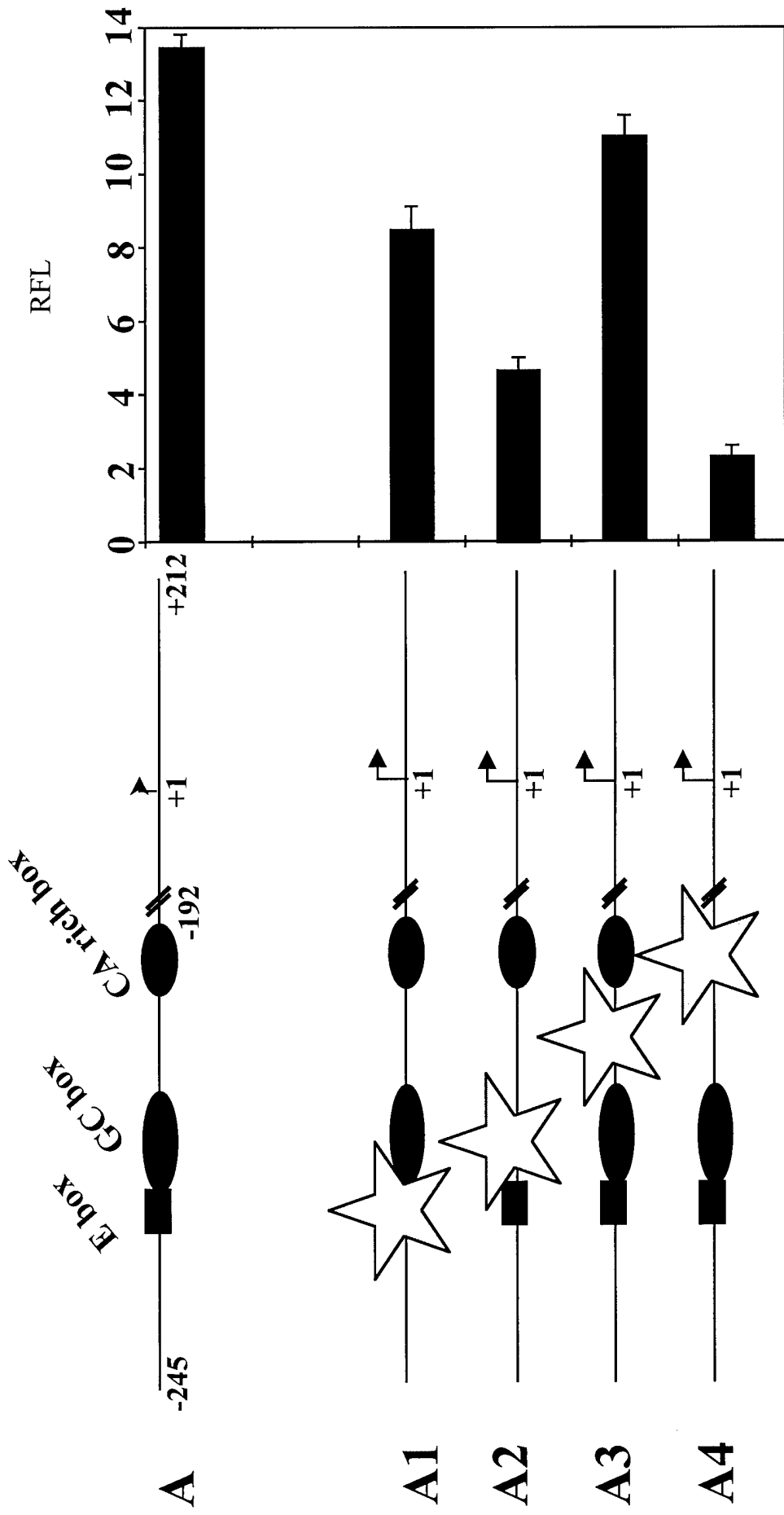


Fig. 4

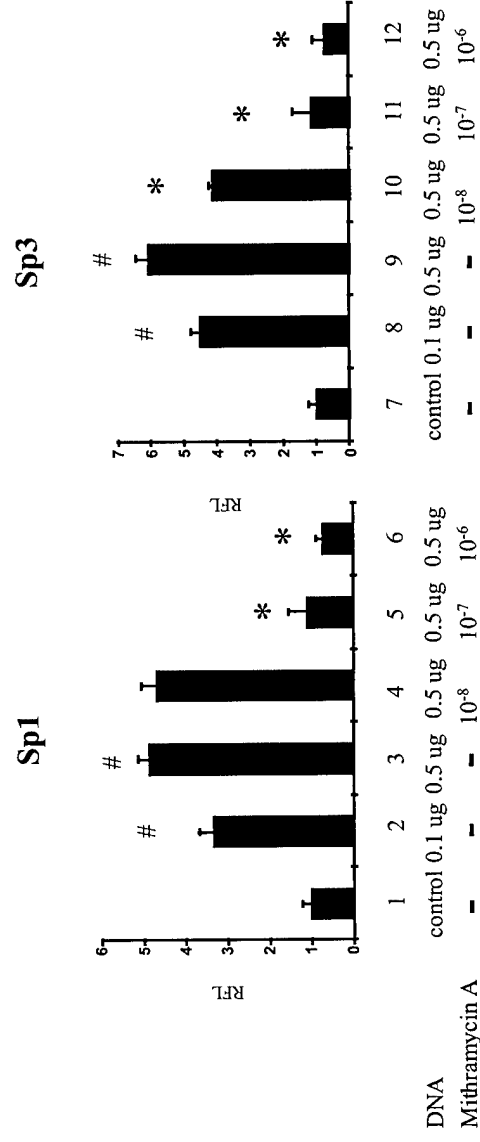
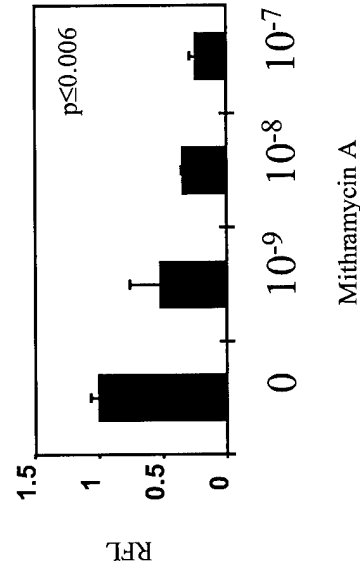


Fig. 6

A



B

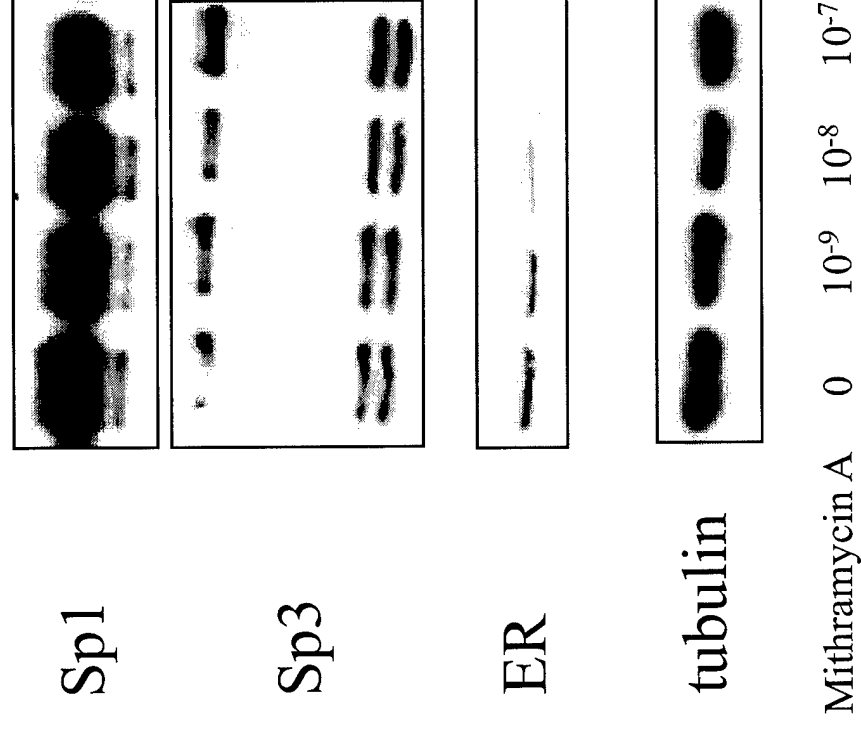


Fig. 7

APPENDIX B

FIGURE LEGENDS:

Fig. 1. Sp1, Sp3, USF-1 and ER α interact with the -245 to -182 bp region of the ER α promoter. A, EMSA was performed using MCF-7 extracts. An end-labeled (20,000 cpm) oligonucleotide which spanned the ER promoter from -245 bp to -182 bp (Appendix A, Fig. 3) was incubated with 30 μ g of extract alone. The complex is indicated by a box. B. ER α promoter-binding proteins isolated by EMSA (A) and MCF-7 extract (B) were resolved by SDS-PAGE and probed with antibodies to Sp1, Sp3, USF-1 and ER α .

Fig. 2. Sp1, Sp3, and USF-1 but not ER α are able to bind to the -245 to -182 bp region of the ER α promoter. EMSAs were performed using *in vitro* translated products. An end-labeled (20,000 cpm) oligonucleotide which spanned the ER promoter from -245 bp to -182 bp was incubated alone (lane A), or in the presence of rabbit reticulocyte (lane B), USF-1 IVT product (lane C), USF-1 IVT with anti-USF-1 antiserum (lane D), USF-1 with rabbit pre-immune serum (lane E), USF-1 IVT with 10-fold molar excess of a cold oligonucleotide containing a consensus USF-1 binding site (lane F), USF-1 IVT with 10-fold molar excess of a cold oligonucleotide containing an Sp1 consensus binding site (lane G), Sp1 IVT product (lane H), Sp1 IVT with an anti-Sp1 antiserum (lane I), Sp1 IVT with rabbit pre-immune serum (lane J), Sp1 IVT with 10-fold molar excess of a cold oligonucleotide containing an Sp1 consensus binding site (lane K), Sp1 IVT with 10-fold molar excess of a cold oligonucleotide containing a consensus USF-1 binding site (lane L), Sp3 IVT product (lane M), Sp3 IVT with an anti-Sp3 antiserum (lane N), Sp3 IVT with rabbit pre-immune serum (lane O), Sp3 IVT with 10-fold molar excess of a cold oligonucleotide containing an Sp1 consensus binding site (lane P), Sp3 IVT with 10-fold molar excess of a cold oligonucleotide containing a consensus USF-1 binding site (lane Q), ER α IVT (lane R), ER α IVT with anti-ER antiserum (lane S). A 32 P-radiolabeled consensus ERE probe was incubated alone (lane T).

with the ER α IVT product (*lane U*) or with the ER α IVT product and anti-ER antiserum (*lane V*). Complexes are indicated by *stars* with supershifted complexes indicated by arrows.

Fig. 3. USF-1 transactivates the ER α minimal promoter in MCF-7 breast cancer cells. MCF-7 cells were co-transfected with the ER minimal promoter luciferase reporter construct and pCDNA3.1 vector (column A) or increasing concentrations of either USF-1 alone (columns B-D) or USF-1 with increasing concentrations of a dominant-negative USF-1 expression vector (columns E and F). Luciferase values were normalized to Renilla luciferase expression. Fold induction was calculated relative to the normalized luciferase activity obtained by transfecting the minimal promoter reporter with the pCDNA3.1 vector alone and was representative of two independent experiments done in triplicate

Fig. 4. Multiple factors transactivate the ER α minimal promoter in MCF-7 cells. MCF-7 cells were co-transfected with the ER minimal promoter luciferase reporter construct and the pCDNA3.1 vector (A), an Sp1 expression vector (B), a USF-1 expression vector (C), an ER α expression vector (D) or a combination of Sp1 and USF-1 (E) Sp1 and ER α (F), USF-1 and ER α (G) or Sp1, USF-1 and ER α expression vectors. Luciferase values were normalized to Renilla luciferase expression. Fold induction was calculated relative to the normalized luciferase activity obtained by transfecting the minimal promoter reporter with the pCDNA 3.1 vector alone and was a combination of three independent experiments done in triplicate.

Fig. 5. Identification of important regulatory nucleotides in the ER promoter by mutagenesis. *Left panel*, schematic presentation of the ER α minimal promoter fragments and the potential transcriptional elements (A), the linker-scanner mutation (star) between nucleotides -203 and -192 (E), and the 4 bp mutations within the -203 to -192 bp region (E-1 – E-3). *Right panel*, promoter activity was expressed as corrected relative fold luciferase units over control and was representative of two independent experiments done in triplicate.

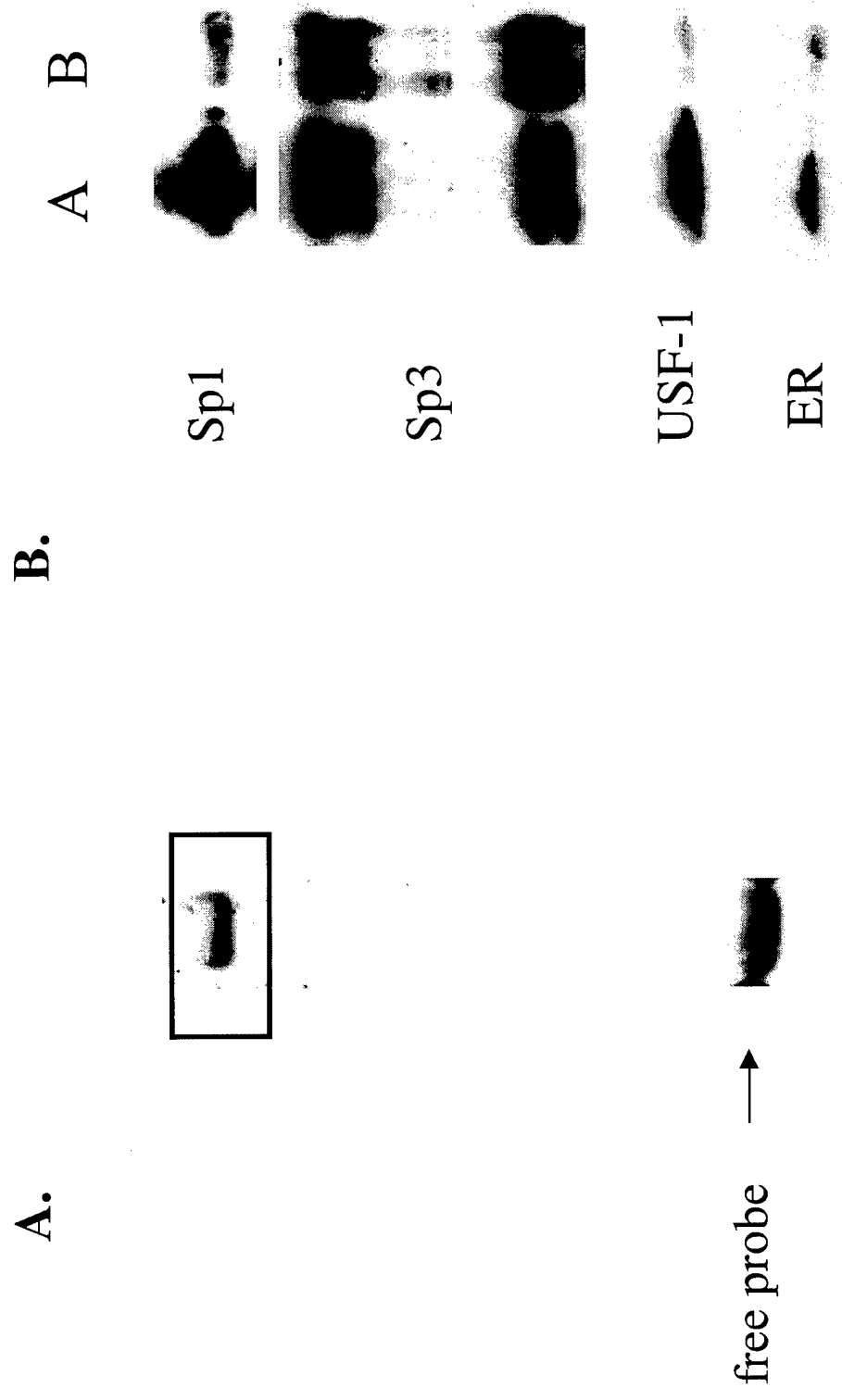


Fig. 1

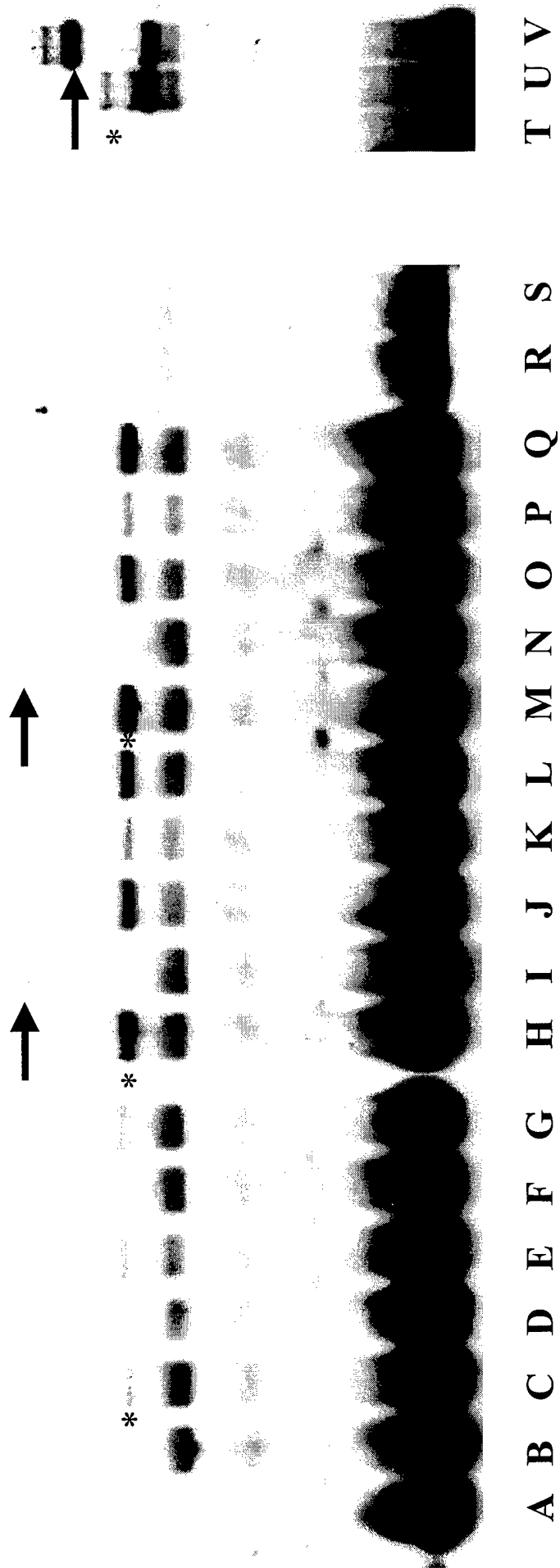


Fig. 2

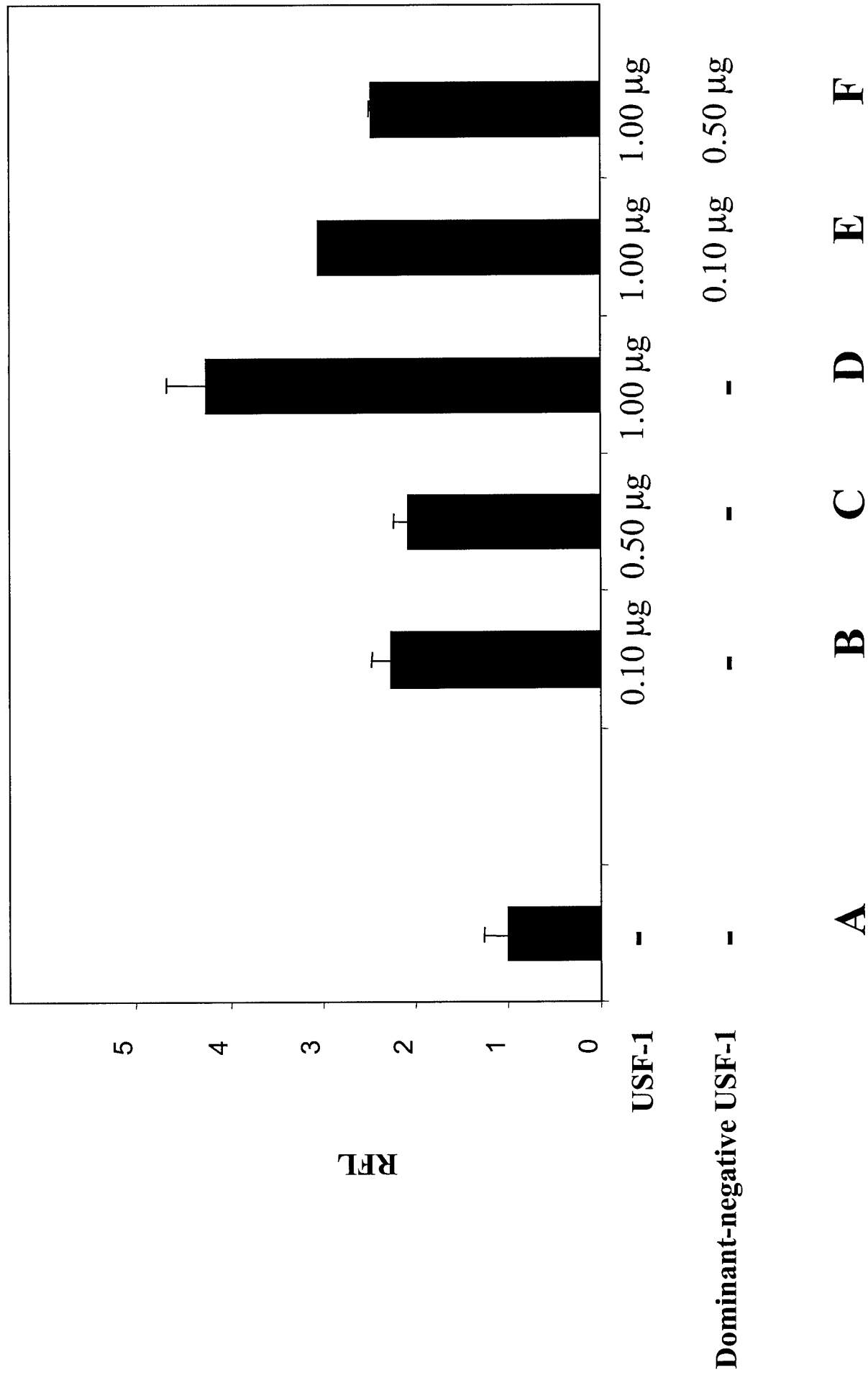


Fig. 3

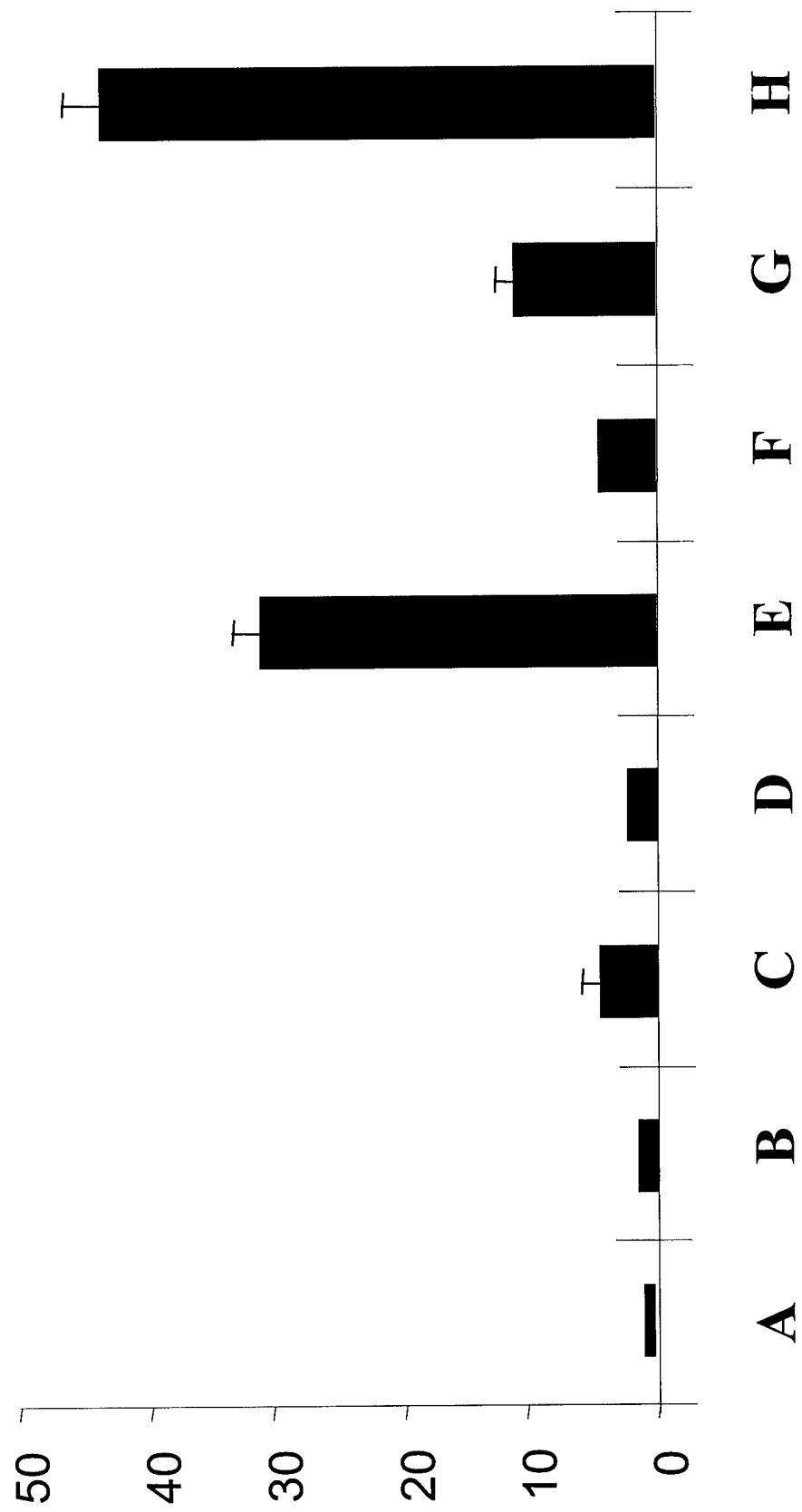


Fig. 4

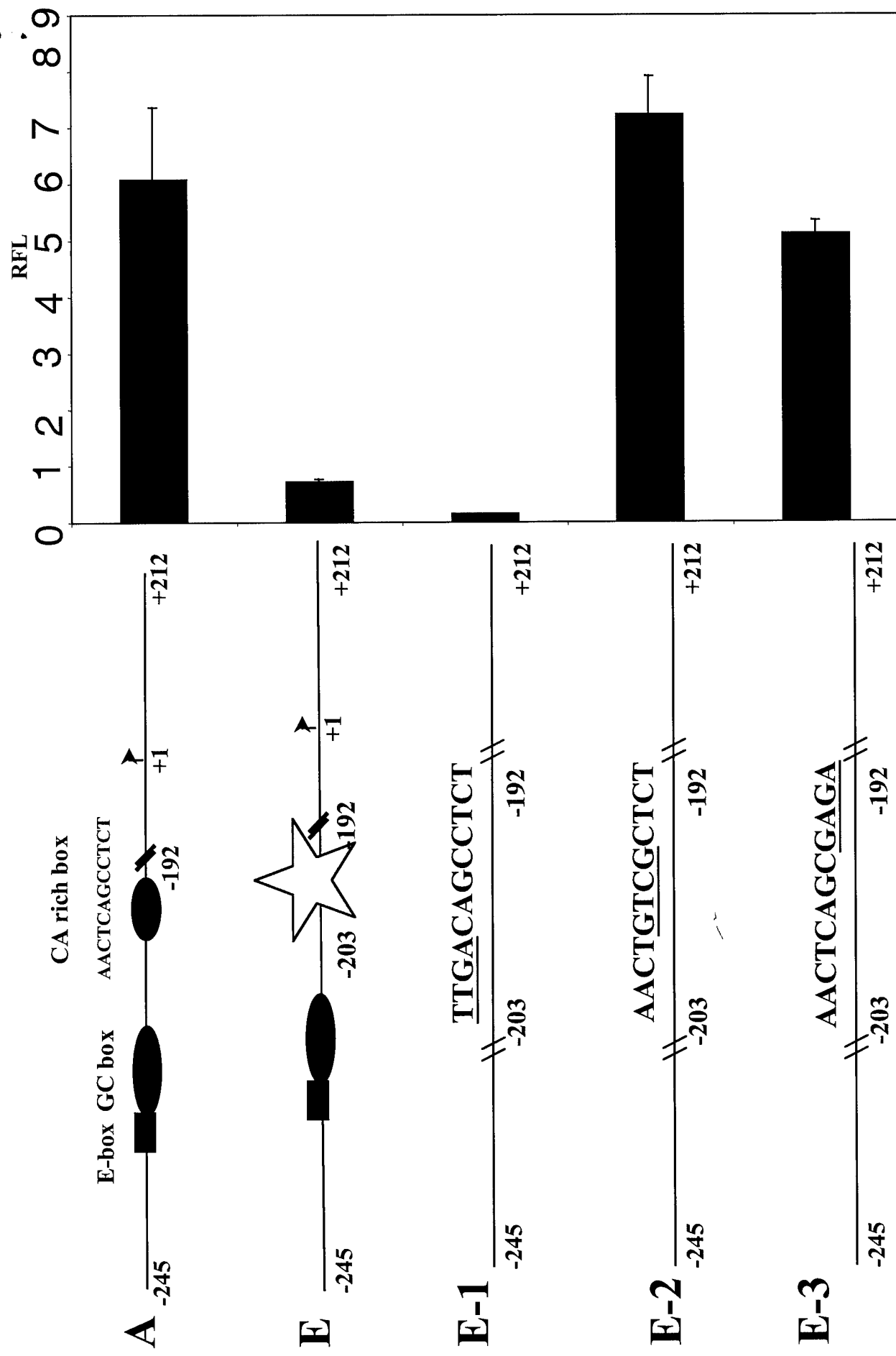


Fig. 5